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Molecular Identification and Immunolocalization of Purinergic Signaling Receptors in the Mammalian Vomeronasal Organ

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MOLECULAR IDENTIFICATION AND THE IMMUNOLOCALIZATION OF PURINERGIC SIGNALING RECEPTORS IN THE MAMMALIAN VOMERONASAL ORGAN

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ABSTRACT

Information about the external world is conveyed by the nervous system via specialized sensory organs such as the vomeronasal organ (VNO). The VNO is crucial for pheromone detection and the regulation of social behavior in many mammals. Recent research has shown that purinergic signaling pathways in the VNO play a role in the chemosensory activity of the organ. There are two families of purinergic receptors that may be involved in this activity, P2X and P2Y. We hypothesized that the vomeronasal sensory neurons express both P2X and P2Y receptors which, when activated by ATP or other purine/pyrimidine nucleotides, work to maintain homeostasis and assist in signal transduction in the tissue. This hypothesis was partially addressed using reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and immunocytochemistry to determine where P2X receptors are expressed and if P2Y receptors are expressed in the mouse VNO. RT-PCR results suggested that there is gene expression of $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2X_1$, and $P2X_3$ receptors in the tissue. A selection of the more commonly expressed purinergic receptors explored with immunohistochemistry indicated that $P2Y_1$, $P2Y_2$, and $P2X_1$ receptors are widely expressed throughout the VNO, including expression in the sensory and non-sensory epithelia. Immunocytochemistry results demonstrated expression of $P2Y_1$ and $P2Y_2$ receptors on the vomeronasal sensory neurons, further supporting our hypothesis that the purinergic receptors play a role in signal transduction. In conclusion, our data strongly support the hypothesis that there are purinergic receptors present in the VNO of mice and are in agreement with our previous data showing purinergic receptor-mediated modulation of chemosensory signal transduction in vomeronasal sensory neurons.

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INTRODUCTION

Pheromone Signaling and the Vomeronasal Organ

Pheromones are a powerful and ubiquitous form of communication and molecular control among species spanning every animal phylum, including single-cell organisms. They are commonly defined as secreted or excreted chemical signals that trigger a behavioral response in another member of the same species (Karlson and Luscher, 1959). Recent research suggests that pheromones also induce endocrine responses, and ongoing study will continue to determine the extent of the impact of pheromone signaling (Dulac and Torello, 2003). Extensive study on the characterization of pheromones has demonstrated that they exist as a wide variety of signaling molecules with many chemical forms, including proteins and other small molecules (Brennan and Zufall, 2006). While they have been classically defined as nonvolatile (unable to disperse as a vapor), recent research has again demonstrated that the functional characteristics may be far more complex, with nonvolatile and volatile forms now being recognized as pheromones (Stowers and Marton, 2005). Two major factors that determine pheromones' volatility in the air and solubility in water are their size and polarity, with the substances often being released in sweat and urine (Karlson and Luscher, 1959, Brennan and Zufall, 2006).

In mammals, the chemosensory structure known as the vomeronasal organ (VNO) contains receptors that are specialized to receive and transmit pheromone signals. The VNO was first identified in 1813 by Danish anatomist Ludvig Jacobson who believed it to be a secretory organ (Doving and Trotier, 1998). Over the past twenty years extensive study has revealed that the VNO is in fact an accessory olfactory organ that is part of the larger olfactory system (Halpern and Martínez-Marcos, 2003). This system includes the

main olfactory epithelium (MOE), the septal organ, and the Grüneberg ganglion, in addition to the VNO (Eisthen, 1997, Halpern and Martínez-Marcos, 2003). Although pheromone detection has been demonstrated in other tissues such as the MOE, in which around 5% of olfactory neurons are responsive to pheromones, the VNO is highly specialized with the vast majority of the vomeronasal sensory neurons (VSNs) responding to pheromones (Halpern and Martínez-Marcos, 2003, Oshimoto et al., 2013). Detailed structural information on the VNO is now available, and its signal transduction mechanisms continue to be elucidated. The critical role it plays in regulating behavior through the transduction of pheromones is also well documented (Halpern and Martínez-Marcos, 2003).

Pheromone signal transduction by the VNO plays an important role in regulating the behavior and activity of mammals. The endocrine responses initiated through pheromone signaling can have long term effects, while behavioral responses are often rapid and transient in nature (Dulac and Torello, 2003). An example of the pheromonal regulation of endocrine functions in mice is the modification of the hypothalamic-pituitary axis upon the chemotransduction of male and female pheromones. When exposed to male pheromones a female mouse will experience an increase in luteinizing hormone and a decrease in prolactin as regulated by the hypothalamic-pituitary axis, while exposure to female pheromones will lead to an increase in prolactin in other female mice (Keverne and de la Riva, 1982, Dulac and Torello, 2003). These changes in endocrine function can lead to the onset of puberty or the termination of pregnancy in female mice upon detection of male pheromones (known as the Bruce Effect), while the detection of female pheromones by female mice can cause a delay in the onset of puberty

(Bruce, 1959, Bellringer et al., 1980). These pheromone regulated behavioral changes initiated by the modification of the hypothalamic-pituitary axis have been named "primer pheromone effects" (Halpern and Martínez-Marcos, 2003).

Pheromone signaling can also lead to rapid behavior modification in mice (Dulac and Torello, 2003). These modifications include the initiation of aggression, copulatory behavior, and lordosis (a posture that demonstrates sexual receptivity), among others (Dulac and Torello, 2003, Chamero et al., 2012). Removal of the mouse VNO severely disrupts reproductive behavior so that mounting and lordosis are not preferentially directed towards the opposite sex (Stowers et al., 2002, Samuelsen and Meredith, 2009). Trpc2 (transient receptor potential cation channel 2) mutants lose some VNO functionality, and along with it aggressive and territorial behaviors (Chamero et al., 2012). The VNO also plays an important role in eliciting predator based defensive behaviors in mice (Stowers et al., 2002, Chamero et al., 2012).

Despite its powerful influence among many mammals, it is widely accepted that humans no longer have a VNO, although we still respond to pheromones through signal reception in the MOE (Halpern and Martínez-Marcos, 2003, Wang et al., 2007, Chamero et al., 2012). Regardless of the presence or absence of the VNO in humans, it remains an important and dynamic organ to study as it provides "fundamental information on the organization and evolution of chemical communication," as well as informing on the neural processing of the historically mysterious sexual and social behaviors of animals (Chamero et al., 2012).

The mouse VNO is a paired tubular structure at the tip of the nose that is encased in a bony capsule (Halpern and Martínez-Marcos, 2003) (Figure 1). The lumen through which substances move separates the non-sensory and sensory epithelia and is connected to the nasal cavity by the vomeronasal duct (Halpern and Martínez-Marcos, 2003). On the side of the non-sensory epithelium a large blood vessel runs the length of the VNO lateral to the lumen (Dulac and Torello, 2003). This section of the tissue is also populated by connective tissue and glands that underlie the ciliated non-sensory epithelium (Dulac and Torello, 2003). The cilium of the non-sensory epithelium beats to help move material in and out of the lumen of the VNO.

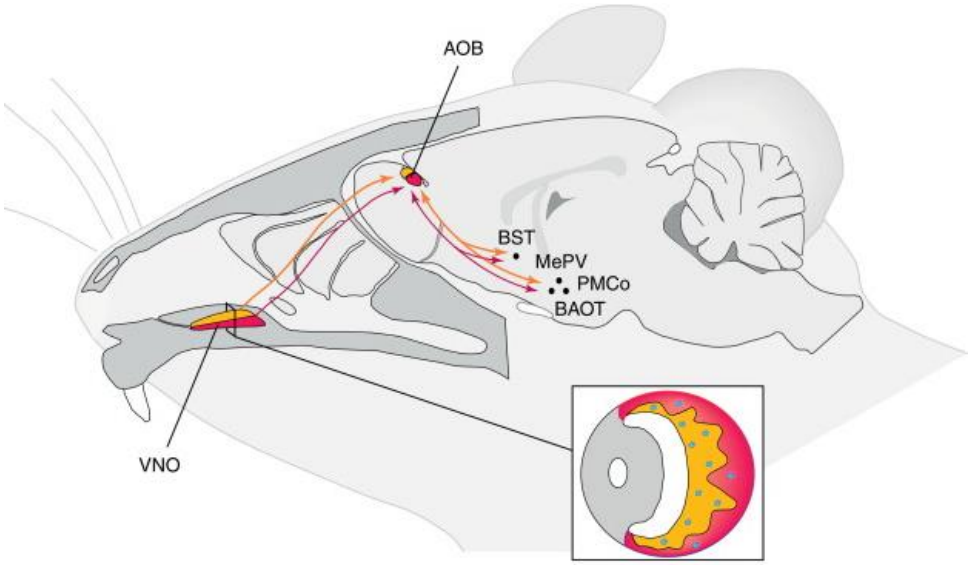

Figure 1. Sagittal view of a mouse head highlighting the location of the VNO and the AOB. Apical and basal VSNs project their axons to the glomeruli of the anterior (yellow) or posterior (red) AOB respectively. In turn, the AOB transmits to the bed nucleus of the stria terminalis (BST), the bed nucleus of the accessory olfactory tract (BAOT), the posteroventral medial amygdala (MePV), and the posteromedial cortical amygdala (PMCo). The inset shows a diagram of a coronal section of the VNO, showing the crescent-shaped sensory epithelium with the two major layers, apical and basal. Reprinted from Trends in Neuroscience, 35(10), Chamero, P., Leinders-Zufall, T., & Zufall, F., From genes to social communication: molecular sensing by the vomeronasal organ, 597-606, 2012 with permission from Elsevier.

Substances arrive in the lumen of the VNO through a mechanism known as the vasomotor pump. Meredith et al. (1980) first demonstrated that the blood vessels in the vicinity of the hamster VNO serve as a pump that draws chemicals into the lumen. In hamsters, this pump appears to be activated in response to any novel stimulus (Meredith, 1994). Ben-Shaul et al. (2010) found that a similar vasomotor pumping activity also occurs in the mouse VNO to draw in potential signaling molecules. This vasomotor pumping mechanism not only draws chemicals into the lumen for detection by the VSNs, but leads to a release of ATP (Vick and Delay, 2012).

The crescent-shaped sensory epithelium, or neuroepithelium, of the VNO is populated by vomeronasal sensory neurons (VSNs), supporting cells, and basal cells (Halpern and Martínez-Marcos, 2003) (Figure 2). VSNs are bipolar cells that have a single apical dendrite and dendritic knob that reaches to the surface of the lumen and an axon that projects to the accessory olfactory bulb (AOB) (Dulac and Torello, 2003, Ghiaroni et al., 2003, Chamero et al., 2012). The AOB is positioned on the dorsal posterioregion of the olfactory bulb (Figure 1) (Chamero et al., 2012). The dendrites of the VSNs end in dendritic knobs with microvilli that contain much of the chemosensory machinery of the VNO including the V1R and V2R receptors and Trcp2 (Chamero et al., 2012). These dendrites are bathed continually in fluid that is secreted from the VNO glands at the ventral and dorsal tops of the lumen (Dulac and Torello, 2003). In isolation, VSNs are identifiable by their large cell body and clear dendrite.

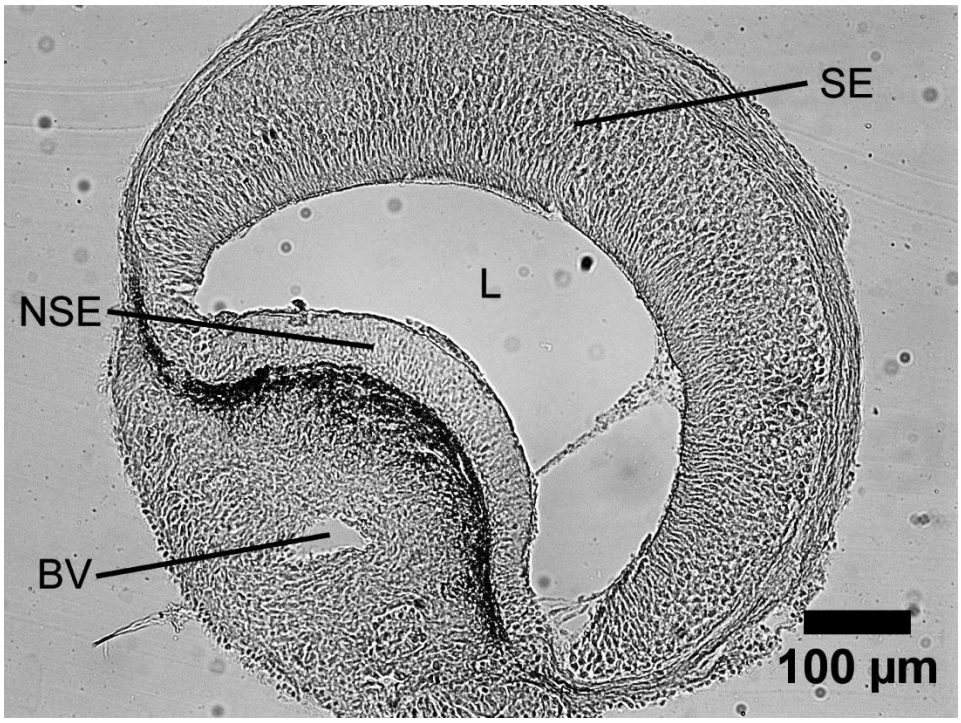

Figure 2. A coronal section of the mouse VNO. The lumen (L), sensory epithelium (SE), non-sensory epithelium (NSE), and the blood vessel (BV) are labeled for reference. Within the sensory epithelium VSNs with dendrites reaching to the luminal surface can be observed. DIC image taken with a 20x lens.

VSNs are noted to activate at current injections as low as 1 pA, which may help account for their high sensitivity to chemical signals (Liman and Corey, 1996, Inamura and Kashiwayanagi, 2000). Signal transduction in the VNO is largely dependent on receptors in the dendritic knobs of the VNO. Upon activation by pheromones, receptors such as V1R (in the apical layer) and V2R (in the basal layer) activate downstream signal transduction mechanisms (Berghard et al., 1996, Holy et al., 2000, Ukhanov et al., 2007). This includes the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC) (Krieger et al., 1999, Dulac and Torello, 2003). The increase in IP_3 leads to a subsequent increase in intracellular Ca^{2+} that is released from intracellular stores and influxes from extracellular sources, while DAG activates Trpc2

which leads to an influx of Ca^{2+} and Na^{+} into the microvilli of the VSNs (Halpern and Martínez-Marcos, 2003). The influx of Ca^{2+} can go on to stimulate calcium-activated chloride current that amplifies the response of the VSNs (Yang and Delay, 2010, Kim et al., 2011). As mentioned earlier, this signal transduction is carried by the axon to the AOB, where the release of glutamate serves as the neurotransmitter to transmit the information throughout the central nervous system (Dudley and Moss, 1995).

The VSNs share the neuroepithelium of the VNO with the supporting cells and the basal cells. The supporting cells of the VNO are bipolar with cell bodies that lie close to the luminal border, with short apical processes that reach the epithelial surface and a long basal process that reaches to the basal lamina (Ghiaroni et al., 2003). It is possible that supporting cells play a role in chemosensory activity of the VNO (Hassenklover et al., 2008). They are notable for possessing voltage-gated ion channels that may play a role in maintaining homeostasis of the tissue and regulating the chemical composition of the intercellular fluid in a manner which supports the excitability of the VSNs (Ghiaroni et al., 2003). The basal cells of the VNO lie along the basal lamina of the neuroepithelium, and replace apoptotic VSNs through migration and differentiation (Cappello et al., 1999, Giacobini et al., 2000). VSNs are continually undergoing apoptosis and being replaced, even in adult animals (Giacobini et al., 2000). This ensures rapid tissue repair following damage from the harsh environmental stimuli the VNO comes in contact with in order to ensure that the tissues maintains optimum performance (Giacobini et al., 2000).

Purinergic Signaling

Purinergic signaling, a form of chemical communication in which a purine, commonly adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP), or adenosine, serves as a signaling molecule through the excitation of P1 and P2 receptors, has become an important area of study as purinergic signaling pathways have been found to be widespread and have versatile functionality among mammals (Hegg CC, 2003, Abbracchio et al., 2009, Housley et al., 2009). Purinergic signaling was first identified in 1972, when it was demonstrated that ATP was a neurotransmitter in the noncholinergic, non-adrenergic inhibitory nerves of the guinea-pig's taenia coli (Burnstock, 1972). Widespread acceptance of the hypothesis that purines could serve as signaling molecules did not occur until the identification and cloning of purinergic receptors in the 1990s (Burnstock, 2009). In the twenty years since, it has been determined that purinergic signaling receptors are among the most abundant signaling receptors in mammals, having been found in every cell and tissue type examined including nervous tissue (Burnstock and Knight, 2004). ATP has been shown to be a transmitter or cotransmitter in nerves throughout the peripheral and central nervous system (Abbracchio et al., 2009). It serves as a fast excitatory neurotransmitter as well as a long-term modulator of growth and development, cell proliferation, and cytotoxicity, making purinergic signaling a critical and influential signaling system (Zimmermann, 2006, Burnstock, 2009).

There are three classes of purinergic receptors*;* G-protein coupled P1 adenosine receptors, ionotropic P2X receptors, and G-protein coupled P2Y receptors (Burnstock, 2009). The adenosine receptors have been further split into four categories, A1, 2A, 2B, and 3 (Burnstock, 2009). The ionotropic P2X receptors are designated $P2X_1$-$P2X_7$

according to the seven distinct genes that code for the individual subunits of the trimer that contains two transmembrane domains and a large extracellular loop (Nicke et al., 1998, North, 2002, Roberts et al., 2006). They are classic ligand-operated ionotropic channels that open upon binding of ATP, with a pore that is permeable to monovalent and divalent cations, including Na^+, K^+ and Ca^{2+} (Ding and Sachs, 1999, Egan et al., 2006). The monomer subtypes can form homomeric or heteromeric receptors, making them a versatile form of receptor (Abbracchio et al., 2009).

While P2X receptors are activated primarily by ATP, P2Y receptors can be activated by ADP, uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), and UDP-glucose (Abbracchio et al., 2009). These G-protein coupled receptors have the classic seven transmembrane domains and are classified into two distinct P2Y subgroups: the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ subgroup and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ subgroup (Abbracchio et al., 2006). The first subgroup consisting of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ typically couples with the G-protein G_q/G_{11} to activate PLC, leading to the release of IP_3 and the subsequent release of intracellular Ca^{2+} from the endoplasmic reticulum (Abbracchio et al., 2009). The second subgroup consisting of $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ couples with G-protein $G_{i/o,}$ which inhibits adenylyl cyclase and subsequently modulates ion channel activity (Abbracchio et al., 2009). These purinergic receptors have been extensively reviewed in (Abbracchio et al., 2009) and (Burnstock, 2009).

Purinergic Signaling in the VNO

Recently the role of purinergic signaling in the VNO has been studied in the lab of Dr. Rona Delay through the use of electrophysiology, with results suggesting that purinergic receptors play important functional roles in the VSNs. Purinergic signaling in

the MOE has been more extensively reported in the literature. P2X and P2Y receptors are expressed in the sensory epithelium of the mouse MOE, which upon reception of ATP reduce the response of the olfactory sensory neurons (OSNs) while also increasing intracellular [Ca^{2+}] (Hegg CC, 2003). The ATP that serves as the signaling molecule in this pathway is released upon exposure of the OSNs to high concentrations of odorants, and induces basal progenitor cells to differentiate into OSNs to replace dead and dying neurons (Hegg and Lucero, 2006, Hassenklöver et al., 2009). This suggests that in the MOE purinergic receptors play a neuroprotective and neuroregenerative role by preventing OSN toxicity and supporting differentiation.

However, the role of purinergic signaling in the VNO appears to be more varied. Vick and Delay (2012) demonstrated that P2X receptors on isolated VSNs are activated by ATP and elicited an inward current as well as a concentration-dependent increase in intracellular [Ca^{2+}](Figure 3). Vick and Delay (2012) also demonstrated that ATP increased the response of VSNs to dilute urine. The study concluded that the release of ATP upon mechanical stimulation of the VNO such as during activity of the vasomotor pump led to P2X activation which may help regulate the chemosensory activity of the organ (Vick and Delay, 2012). The release of purine nucleotides in a mechanosensative manner has been well documented in many other tissues, including the retina, urinary bladder, kidney nephron, epithelium of the lung, and the cochlea (Homolya et al., 2000, Newman, 2001, Zhao et al., 2005, Sipos et al., 2009, Chen et al., 2010). While Vick and Delay (2012) found that P2X receptor activation led to an increase in [Ca^{2+}]i and an inward current, the activation of P2Y receptors did not elicit a similar response. However, the possibility remained that these purinergic receptors were present in the organ, and

performing a yet unrecognized function. This hypothesis was supported by recent unpublished data from Vick and colleagues that indicated a functional role for P2Y in enabling persistent firing of VSNs (Figure 4).

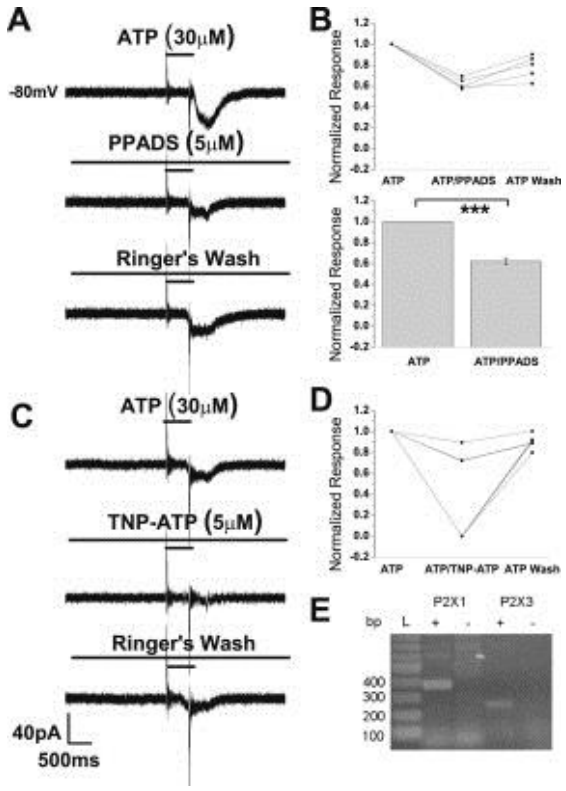

Figure 3. Electrophysiological data from isolated VSNs demonstrating the role of P2X receptors in generating the inward current elicited by ATP. (A) The partial block of the inward current by PPADS indicated the P2X receptor is responsible for the generation of the current. The normalized inward currents for each VSN tested for this response can be seen in (B). (C) The block of the inward current with TNP-ATP again indicated that the P2X receptor is responsible. The normalized inward currents for each VSN tested are plotted in (D). (E) RT-PCR demonstrated the gene expression of $P2X_1$ receptors (382 bp) and $P2X_3$ receptors (252 bp) in the VNO. L = ladder, + = experimental groups, − = control groups. Reprinted with permission from Neuroscience, 220, Vick J.S. and Delay R. J., ATP excites mouse vomeronasal sensory neurons through activation of P2X receptors, 341-350, 2012.

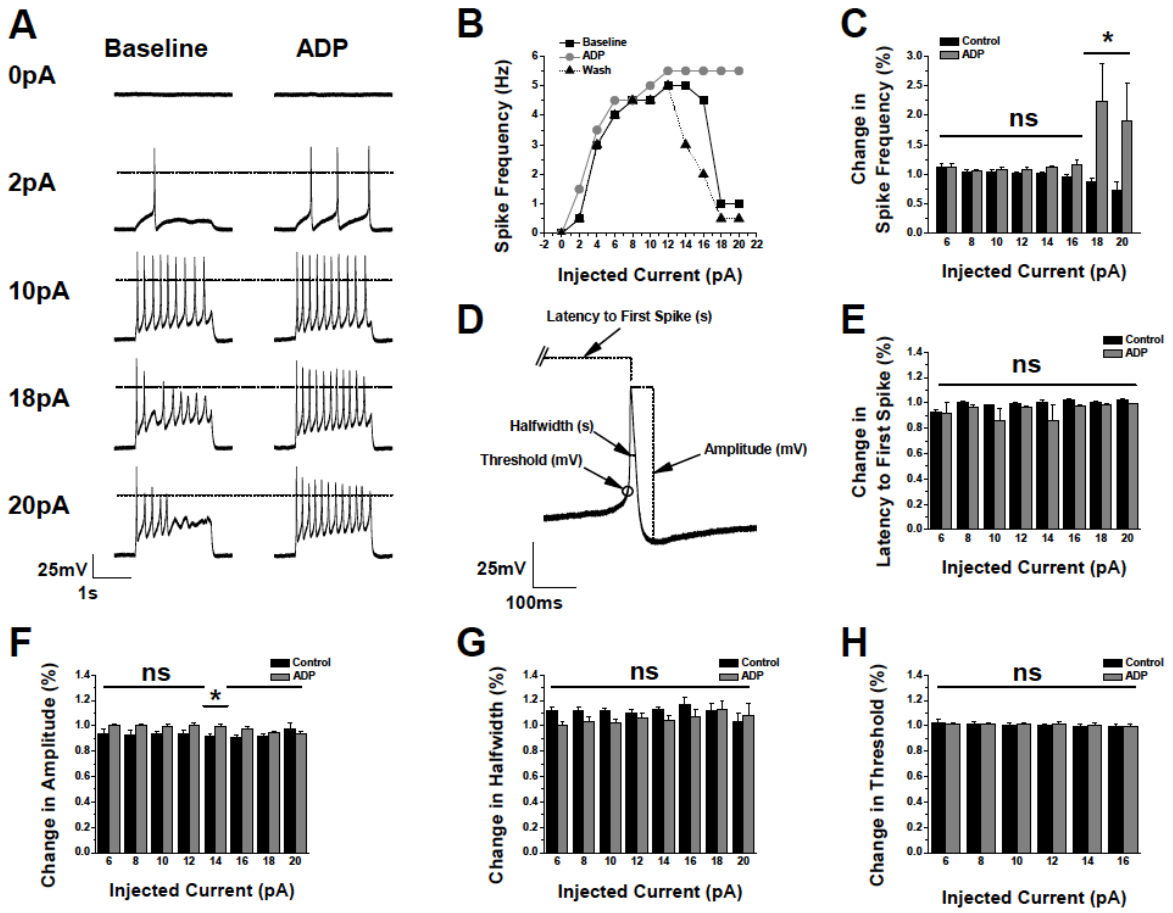

Figure 4. The $P2Y_1$ agonist ADP increased spike frequency in isolated VSNs at relatively depolarized current injections without altering other characteristics of the action potential. (A) The increase of spike frequency upon the application of ADP can be clearly seen relative to baseline. (B) A quantification of the spike frequency from the VSN in (A) revealed a reversible stabilization of the maximal rate of firing at higher current injections. (C) The difference in the average percentage change in spike frequency of control and ADP was statistically significant at 18 to 20pA of current injection. (D) The schematic used to analyze each action potential's amplitude, latency, halfwidth, and threshold voltage with a custom made script written in MatLab by Roman Popov. (E)-(H) The difference in the average percentage change in latencies to first spike, amplitudes, halfwidths, and the thresholds of the control and ADP were not statistically significant. Figure reprinted with permission from Jonathan Vick.

Working from this data, the study herein aimed to continue the exploration of purinergic receptors in the VNO by identifying gene expression of P2Y receptors in the tissue and using methods of immunohistochemistry and immunocytochemistry to

determine the immunolocalization of selected common P2Y and P2X receptors. Immunohistochemistry in the literature has demonstrated that subsets of both receptors have immunoreactivity with the VNO, but no immunocytochemistry has confirmed in what cell types the expression occurs (Gayle and Burnstock, 2005). We hypothesized that P2X and P2Y receptors would demonstrate gene expression in the VNO with immunolocalization to the sensory epithelium, where ATP release via activity of the vasomotor pump would activate the purinergic receptors and lead to subsequent modulation of the VSN response. The immunolocalization and activity of this pathway in the VNO would implicate purinergic signaling in the regulation of mammalian social and sexual behaviors.

MATERIALS AND METHODS

VNO Dissection

All experiments were performed with C57 BL/6 and BALB/C mice raised and euthanized in accordance with the University of Vermont's Institutional Animal Care and Use Committee under IACUC protocol 07-094. Euthanasia was accomplished through CO_2 asphyxiation followed by cervical dislocation. After the animal was sacrificed the VNO was immediately removed through dissection. The head was removed from the body, cut bilaterally, and the VNO in the bony casing was extracted. The bony casing was then removed in the presence of enzyme free dissociation solution (in mM: 140 NaCl, 10 HEPES, 10 Glucose, 5 KCl, 2 EDTA, pH. 7.4).

Reverse Transcription Polymerase Chain Reaction

To identify gene expression of selected P2Y receptors in the VNO reverse transcription polymerase chain reaction (RT-PCR) was performed using primers for $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. Primers were obtained from Integrated DNA Technologies with sequences from del Ray et al. (2006) (Table 1). Total RNA was isolated and purified from the VNO using Qiagen's RNeasy Mini Kit according to manufacturer's instruction. The purity of the isolated RNA was tested using a NanoDrop Spectrophotometer courtesy of the Vermont Genetics Network Proteomics Facility.

cDNA was synthesized with 200U SuperScript III Reverse Transcriptase (Invitrogen) and 2.5 μM random hexamers (Applied Biosystems) with 2 μg of total RNA in a volume of 20 μL. RNaseOUT (Invitrogen) was added to the reactions to prevent degradation of the RNA by inhibiting ribonuclease. A negative control was performed by

omitting SuperScript III Reverse Transcriptase in order to ensure that the PCR products were produced from the cDNA rather than contaminants. The following conditions were followed for cDNA synthesis: i. 25° C for 5 minutes, ii. 50° C for 30 minutes, iii. 55° C for 30 minutes, iv. 70° C for 15 minutes, v. final hold at 4 ° C.

PCR was performed using 1 μL cDNA, 12.5 pmol forward and reverse primers, 2.5 mM $MgCl_2$ (Invitrogen), 200 μM dNTPs (New England BioLabs), 10x PCR reaction buffer (Invitrogen), and 1.25U Taq polymerase (Invitrogen) in a final volume of 25 μL. The following conditions were followed for PCR: i. serial heating at 94° C for 5 minutes; ii. amplification over 35 cycles at 94° C for 30 seconds and 72° C for 30 seconds; iii. final hold at 4 °C.

The amplified products were resolved with gel electrophoresis with a 1% agarose gel run at 130 mV for 30-60 minutes. The gel was stained with ethidium bromide for 30-60 minutes and observed and photographed using a UV light box. PCR products were cut out and purified from the agarose gels using Omega Bio-Tek's Gel Extraction Kit according to manufacturer's instructions and sequenced with the assistance of the Vermont Genetics Network (http://www.vermontcancer.org/). Sequences were checked with the basic local alignment search tool (BLAST) to confirm the product was that of the desired purinergic receptor.

Immunohistochemistry

Immunohistochemistry was performed using antibodies for $P2Y_1$, $P2Y_2$, and $P2X_1$ receptors as specified in Table 2. The dissected VNO was fixed in 4% paraformaldehyde fixative in 0.1 M phosphate buffer solution for 1 hour and then rinsed twice in 0.1 M

phosphate buffer. All rinses were accomplished by moving the tissue between the wells of a 6-well plate. The VNO was then embedded in 15% gelatin in 0.1 M phosphate buffer and returned to the 4% paraformaldehyde fixative for 1 hour. The tissue was then washed 6 times for 10 minutes each in 0.1 M phosphate buffer. In order to confer cryoprotection the tissue was run through a graded sucrose solution consisting of 0.5 M, 1 M, 1.5 M, and 2 M sucrose in 0.1 M phosphate buffer solution. The VNO was sectioned with a Microm HM505E cryostat at ~ –26° C to a thickness of 20 μm. Sections were placed in phosphate buffered saline (PBS).

Selected sections were then transferred to wells of a 6-well plate, 4 sections to each well, and washed in a preincubation buffer consisting of 6.5 % normal goat serum in 0.1 M phosphate buffer with 10% Triton-x-100 (Acros Organics) for 1 hour with mixing at room temperature. The sections were then incubated in primary antibody for 24 hours at room temperature with mixing. All antibodies were diluted in preincubation buffer. The $P2Y_1$ primary antibody was diluted to a concentration of 1:100, the $P2Y_2$ primary antibody was diluted to a concentration of 1:500, and the $P2X_1$ primary antibody was diluted to a concentration of 1:500, as detailed in Table 2. A negative control was performed by incubating without primary antibody.

After primary incubation the sections were washed with PBS 6 times for 10 minutes each. They were then incubated with secondary goat anti-rabbit TRITC conjugated antibody (Invitrogen) at a concentration of 1:1000 for 1 hour. The sections were incubated in the dark at 4°C. Sections were washed with PBS and mounted with Flouromount-G (Southern Biotek). Slides were stored in the dark at room temperature for 24 hours to allow the Flouromount to harden and then stored in the dark at 4^0 C.

Sections were imaged with a Zeiss Axioskop2 microscope fitted with a Photometrics CoolSnap EZ camera with NIS Elements used for image capture. Images were obtained using 20x and 40x objectives, with care being taken to image control sections with the same exposure as the experimental sections. All images were saved as TIFF files and processed using the image processing package FIJI, also known as Image J. Edited images were then saved as both TIFF files and JPEG files and a figure was made using Adobe Photoshop.

Immunocytochemistry

Immunocytochemistry was performed using antibodies for $P2Y_1$ and $P2Y_2$ receptors as specified in Table 2. Isolated cells were obtained through either a collagenase or papain digestion. The collagenase digestion was performed by mechanically dissecting the VNO for 10 minutes in dissociation solution supplemented with 1 mg/ml collagenase (Sigma) and 40 μL trypsin (Life Technologies). The digestion solution was rotated for 1 hour before being transferred to a 1 mL eppendorf tube and centrifuged at 4.8 RPM for 1 minute. The resulting cell pellet was then resuspended with Ringer's solution (in mM: 138 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.4) supplemented with 1 mg/mL DNase (Sigma) and rotated for an additional 20 minutes. The resulting cell suspension was transferred to a test tube and gently triturated for 2-3 minutes. The papain digestion was performed by placing the VNO in dissociation solution supplemented with 10mg/mL papain (Calbiochem) for 10 minutes of mechanical dissection. The solution was then transferred to a test tube containing Ringer's solution supplemented with ≈10 μg/mL leupeptin (USB) to inhibit papain and was gently triturated for 2-3 minutes.

The resulting cell suspension from either digestion was plated on round glass cover slips coated with concanavalin A (Calbiochem). The cell suspension was allowed to settle for 30 minutes before being fixed with 4% paraformaldehyde fixative in 0.1 M phosphate buffer for 15 minutes. The cover slips were then rinsed with 0.1 M phosphate buffer. Once plated, the cells were washed with preincubation buffer for 30 minutes. The cells were then incubated with primary antibody for 24 hours at 4° C. $P2Y_1$ and $P2Y_2$ receptor primary antibodies were diluted in preincubation buffer to a concentration of 1:500 (Table 2). A negative control was performed by incubating without primary antibody. After primary incubation the cells were washed with PBS 3 times for 5 minutes each. Secondary incubation was then performed in the dark at 4° C with a secondary goat anti-rabbit TRITC conjugated antibody (Invitrogen) at a dilution of 1:1000 for 1 hour. The cells were then washed with PBS 2 times for 5 minutes each. A final wash in distilled water was then performed before the cover slips were mounted with Flouromount-G to avoid crystallization. The slides were stored in the dark at room temperature for 24 hours to allow the Flouromount to harden before being stored at 4°C.

Isolated VSNs were imaged with a Keyence BZ-9000 Fluorescence microscope with the associated BZ-II Analyzer. VSNs were identified by morphological characteristics using phase contrast microscopy. Images were obtained using a 40x objective with 68x magnification, with care being taken to image control slides with the same exposure as the experimental slides. All images were saved as TIFF files and a figure was made with Adobe Photoshop. Slide screening was also performed at 40x magnification, with 20-30 VSNs being observed for each treatment.

Name	Primer	Sequence	Approximate Product Size (bp)
P2Y1	Forward	5'- ATC AGC GCA CAC AGG TAC AG -3'	200
	Reverse	5'- AAT CAT TGG ACG TGG TGT CA -3’	
P2Y2	Forward	5'- CAG GCC TGT GCA TAT GTG AG -3'	200
	Reverse	5'- GGC AAC AGC ACG TAC TTG AA -3'	
P2Y4	Forward	5'- CCA CAT CAG GGG GAA CTA AG -3'	200
	Reverse	5'- CAA GGA GTC TGC ACT GGT CA -3'	
P2Y6	Forward	5'- AAC CTG CCT TGA AAA CAA CG -3'	200
	Reverse	5'- TTA GCA GCA GTC GCT TGA AA -3'	

Table 1. Primers for RT-PCR and their product sizes in base pairs (bp).

Antigen	Host	Immunohistochemistry Dilution	Immunocytochemistry Dilution	Source
Primary Antibody				
$P2Y_1$ Receptor	Rabbit	1:100	1:500	Alomone Labs
$P2Y_2$ Receptor	Rabbit	1:500	1:500	Alomone Labs
$P2X_1$ Receptor	Rabbit	1:500	N/A	Alomone Labs
Secondary Antibody				
Goat Anti-Rabbit TRITC Antibody	Goat	1:1000	1:1000	Invitrogen

Table 2. List of antibodies for immunohistochemistry and immunocytochemistry.

RESULTS

$P2Y_1$, $P2Y_2$, and $P2Y_6$ receptor transcripts are present in the mouse VNO

We performed RT-PCR in order to determine which P2Y receptor subtypes have gene expression in the mouse VNO using primers for $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. Gene expression of $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors was detected (Figure 5). We observed the product band for each purinergic receptor at the expected locale of 200 base pairs. There was no gene expression of the $P2Y_4$ receptor detected (data not shown). Gene expression was confirmed with sequencing by the Vermont Genetics Network and a basic local alignment search (BLAST). The BLAST results confirmed sequencing with 98%-100% matching identities (Supplemental Figure 1-3).

While the RT-PCR clearly indicated gene expression of the P2Y receptors, there were two anomalies. The first is the band which can be observed at the bottom of the $P2Y_1$ gel (Figure 5). This does not indicate gene expression, but is rather a common by-product of the PCR process known as a primer dimer. The other occurrence is the second band present around 450 base pairs in the $P2Y_2$ gel (Figure 5). This band was sequenced and found to be mucin 5 subtype B, a gene that encodes for a mucosal protein unrelated to purinergic signaling receptors. As the product band in the same gel at 200 base pairs was sequenced and confirmed to be of the $P2Y_2$ receptor, the presence of the mucin 5 subtype B band did not interfere with the results. When taken in conjunction with the results of the RT-PCR performed by Vick and Delay (2012), there is now supporting evidence for gene expression of $P2X_1$, $P2X_3$, $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors in the

mouse VNO. These results support the hypothesis that there is purinergic receptor expression in the mouse VNO.

Localization of purinergic receptors to the sensory epithelium of the VNO

The sensory epithelium of the VNO showed strong immunoreactivity to the $P2Y_1$, $P2Y_2$, and $P2X_1$ receptor antibodies with immunohistochemistry (Figure 6). The $P2Y_1$ receptor antibody exhibited immunoreactivity throughout the sensory epithelium, with particularly intense staining at the apical edge and within the supporting cell layer. The dendrites and the dendritic knob region of the sensory epithelium appeared to be particularly immunoreactive. The cell bodies of the VSNs were also clearly immunoreactive. However, as indicated by the dotted appearance of the staining throughout the sensory epithelium where the cell bodies of the VSNs are located, the $P2Y_1$ receptor antibody did not stain all VSNs but rather demonstrated selectivity. The lack of immunoreactivity in the negative control for the $P2Y_1$ receptor antibody suggested that the staining observed in the sensory epithelium was specific.

The $P2Y_2$ receptor antibody exhibited more uniform immunoreactivity with strong staining throughout the sensory epithelium of the VNO (Figure 6). The dendritic knobs at the apical edge again showed the most intense labeling, with the VSN cell bodies showing strong and consistent staining throughout the tissue sections. The basal lamina region also showed significant immunoreactivity to the $P2Y_2$ receptor antibody. Of particular interest was the staining pattern in the supporting cell layer of the sensory epithelium which displayed a unique pattern of speckling upon close inspection. The

negative control for the $P2Y_2$ receptor antibody suggested that the staining observed in the sensory epithelium was specific.

The immunohistochemistry for the $P2X_1$ receptor antibody also displayed immunoreactivity throughout the sensory epithelium (Figure 6). There was staining present throughout the cell bodies of the VSNs, while the dendritic regions of the epithelium again demonstrated the most intense labeling. However, there was minimal immunoreactivity in the basal lamina and supporting cell layers. The negative control for the $P2Y_2$ receptor antibody again indicated that staining observed in the sensory epithelium was specific.

Localization of purinergic receptors to the non-sensory epithelium of the VNO

In addition to the immunoreactivity that was present throughout the sensory epithelium, there was also immunoreactivity for the $P2Y_1$, $P2Y_2$, and $P2X_1$ receptor antibodies present on the ciliated non-sensory epithelium (Figure 6). The immunoreactivity was uniform throughout the epithelium for all three receptor antibodies, and the negative controls indicated that the staining was specific. It is probable that purinergic receptors, as has been demonstrated by their ubiquitous presence in every cell and tissue type so far examined, are also present in that non-sensory epithelium of the VNO.

Localization of purinergic receptors to the glandular tissue of the VNO

Similar to the staining observed on the non-sensory epithelium, there was strong immunoreactivity for the $P2Y_1$, $P2Y_2$, and $P2X_1$ receptor antibodies in the glandular tissue of the VNO (Figure 6). However, the negative controls for the $P2Y_1$, $P2Y_2$, and

$P2X_1$ receptor antibodies indicate that the staining may be largely nonspecific.

Localization of purinergic receptors to the VSNs

Immunocytochemisty was performed with $P2Y_1$ and $P2Y_2$ receptor antibodies based on antibody availability. Twenty to thirty isolated VSNs were identified morphologically by a rounded cell body, elongated dendrite, and a dendritic knob for each antibody tested and representative isolated VSNs were imaged (Figure 7). Strong immunoreactivity was noted in approximately 60%-70% of the VSNs observed. Immunoreactivity was observed in the cell body, dendritic knob, and dendrite for both the $P2Y_2$ and $P2Y_1$ receptor antibodies. The negative controls show a lack of immunoreactivity.

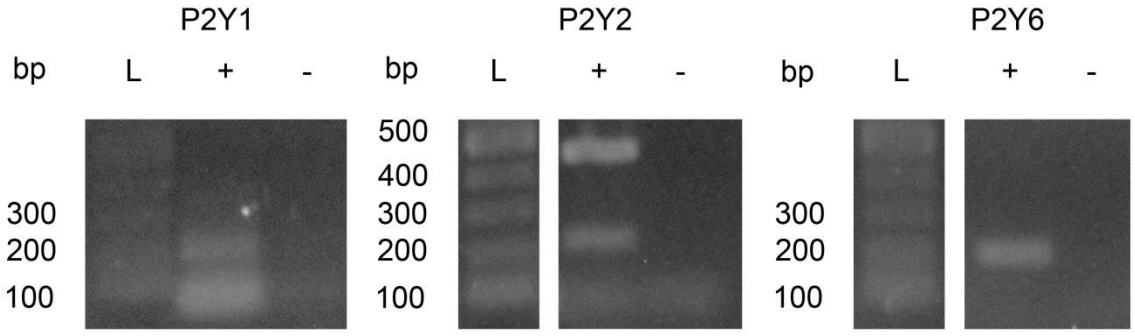

Figure 5. RT-PCR demonstrating gene expression of $P2Y_1$, $P2Y_2$, and $P2Y_6$ in the mouse VNO. The expected product size for all primers was approximately 200 base pairs (bp). L = ladder, + = experimental groups, − = control groups.

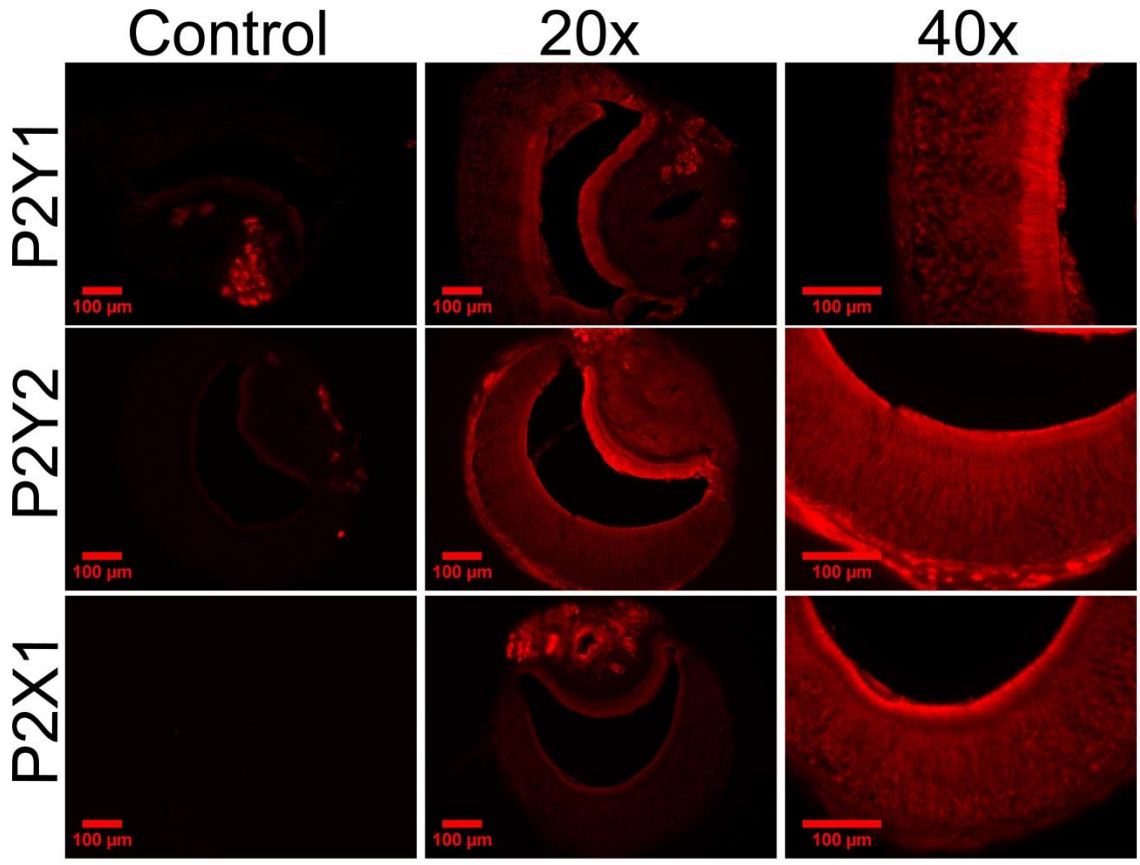

Figure 6. Immunohistochemisty demonstrating the immunoreactivity to $P2Y_1$, $P2Y_2$, and $P2X_1$ receptor antibodies in the mouse VNO. Images were obtained at 20x and 40x magnification with a TRITC filter. Negative controls are also shown.

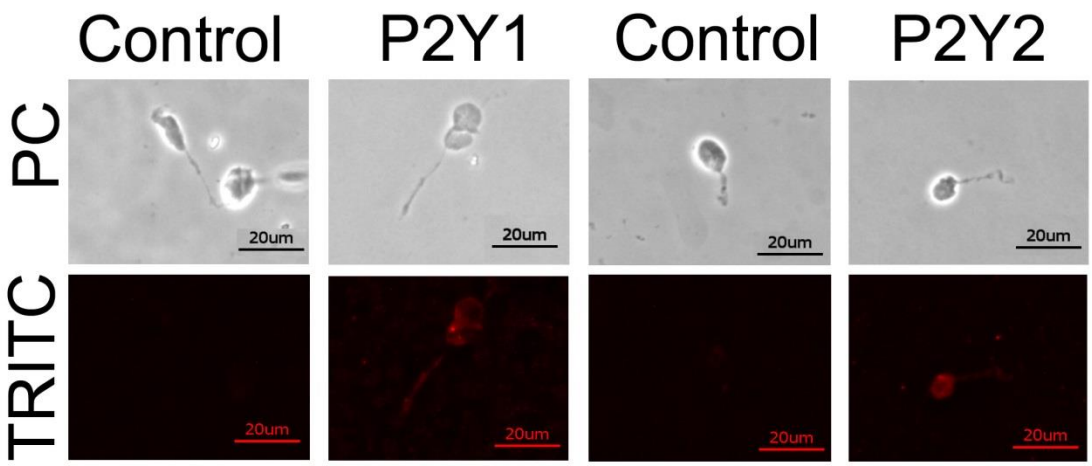

Figure 7. Immunocytochemisty demonstrating the immunoreactivity to $P2Y_1$ and $P2Y_2$ receptor antibodies in isolated VSNs. VSNs were identified by morphology. Images were obtained at 68x magnification with phase contrast (PC) and a TRITC filter. Negative controls are also shown.

DISCUSSION

My results strongly support the hypothesis that there are purinergic receptors demonstrating gene expression and immunolocalization in the mouse VNO. The localization of purinergic receptors to the sensory epithelium of the VNO suggests a role for purinergic signaling that is supported by previous electrophysiological data (Vick and Delay, 2012). The results of the RT-PCR have supplemented the list of purinergic receptors that have been demonstrated to have gene expression in the VNO of mice. While $P2X_1$ and $P2X_3$ receptors were shown to have gene expression in the VNO by Vick and Delay (2012), this study was able to add $P2Y_1$, $P2Y_2$ and $P2Y_6$ to that list. This is the first RT-PCR evidence that P2Y receptors are present in the VNO, supporting our hypothesis and suggesting the possibility that there are a wide range of purinergic receptors present in the organ. To the best of our knowledge, this is also the first study to demonstrate the immunolocalization of purinergic receptors to the VSNs, with the localization of P2Y receptors to the cell bodies and dendritic regions. The immunolocalization of the purinergic receptors to the VSNs and throughout the tissue of the VNO indicates that purinergic receptors may play a wide range of functional roles in the organ, including modulation of pheromone sensory detection and transduction, motilary function, mucocous secretion, and the maintenance of homeostasis.

The immunolocalization of the purinergic receptors $P2Y_1$, $P2Y_2$, and $P2X_1$ to the sensory epithelium of the VNO supports the hypothesis that purinergic signaling receptors in the VNO play a role in the chemosensory activity of the organ. The immunocytochemistry results that demonstrate immunolocalization of $P2Y_1$ and $P2Y_2$ receptors to the VSNs further support this hypothesis. The results closely match what

Gale and Burnstock (2005) observed, with immunoreactivity of the VNO to the $P2Y_1$ and $P2Y_2$ receptor antibody. They saw particularly strong staining in the luminal face of the VNO as we did, as well as staining that extended through the entire epithelium. The immunolocalization of the purinergic signaling receptors to the sensory epithelium and VSNs strongly suggests that they play a role in signal transduction. Interestingly, the receptors are seen to be on the cell body as well as the dendrite, while the V1R/V2R receptors are localized to the dendritic knob. This suggests that the purinergic receptors may be playing a role in maintaining the homeostasis of the cell through activity in the cell body, which modulates it excitability and responsiveness to pheromone signaling. As hypothesized by Vick and Delay (2012), it is also possible that the purinergic receptors, particularly those immunolocalized to the dendritic knob of the VSNs, respond to nucleotides released upon activation of the vasomotor pump that draws material into the lumen of the VNO. The activation of the purinergic receptors may prime the VSNs for pheromone signal transduction, or perhaps work to maintain optimal operating conditions for VSN activity upon the reception of material in the lumen of the VNO by way of the vasomotor pump. Further study is necessary to fully understand this effect.

The immunolocalization of the purinergic receptors to the nonsensory epithelium, particularly the ciliated epithelium and the glandular epithelium, illustrates the multiple functional roles of purinergic receptors. These results suggest that in the VNO alone they may play multiple functional roles, aiding in the chemosensory activity of the organ both through movement of the signaling molecules into and through the lumen and in the VSNs themselves, modulating and maintaining signal transduction. Gayle and Burnstock (2005) also observed that the purinergic receptor immunoreactivity was not limited to the

sensory epithelia. The presence of immunoreactivity of $P2Y_1$, $P2Y_2$, and $P2X_1$ in the ciliated epithelium of the VNO suggests that the purinergic receptor may play a role in the locomotion of cilia and the movement of signaling molecules into and around the lumen of the organ, in order to ensure contact with the sensory epithelium. An increase in ciliary activity was noted in frog epithelium upon contract with increasing concentrations of ATP (Ovadyahu et al., 1988). Similarly, Winters et al. (2008) found that ciliary beat frequency was increased upon activation of $P2Y_2$ receptors in the mouse trachea. When the vasomotor pump is activated and ATP is released, it is possible that activation of the purinergic receptors on the ciliated epithelia results in increased activity in a similar manner. This would conserve energy by ensuring that the ciliated epithelium is only active when needed to move fresh material into and around the lumen of the VNO.

The expression of purinergic receptor antibodies in the glandular tissue of the VNO may also be significant. Not only does it further highlight the extreme diversity of function that the purinergic receptors encompass, but it is also suggests that purinergic signaling in the glandular tissue has an impact on the activity of the VNO. The glandular tissue of the VNO secretes mucous that helps move the pheromone signaling molecules throughout the lumen and lubricates the epithelia (Halpern and Martínez-Marcos, 2003). However, a study of the rat VNO hypothesized that the glandular tissue of the VNO could also be modulating sensory responses through endocrine functions (Lee et al., 2008). Lee et al. (2008) found signaling proteins and mechanisms in the glandular tissues of the VNO, suggesting that the glandular tissues may modulate the chemosensory activity of the VNO by altering the responsiveness of the VSNs in correlation with hormonal status. It is possible, although much study would need to be performed to

address the hypothesis, that the purinergic receptors immunolocalized to the glandular tissue of the VNO may play a role in the chemosensory activity of the tissue beyond excretion of mucus to lubricate the epithelia and transmission of pheromones.

Additionally, the immunoreactivity of the P2Y receptor antibodies to the supporting cell layer of the VNO is indicative that the supporting cells are expressing purinergic signaling receptors in addition to the VSNs. In many other areas of the nervous system, glial and supporting cells have been demonstrated to play an active role in purinergic signaling pathways, expressing many of the receptors (Neary et al., 1996, Brass et al., 2012, Vitanova and Kupenova, 2014). In the MOE, supporting cells express purinergic receptors that upon activation by ATP leads to Ca^{2+} responses and gap junction opening (Vogalis et al., 2005, Czesnik et al., 2006). It has already been suggested that the supporting cells of the vomeronasal organ play an important role in the chemosensory activity of the VNO by potentially mopping-up excess K^+ and Na^+ in the lumen and sensory epithelia in order to maintain homeostasis and optimal VSN activity (Ghiaroni et al., 2003). With this evidence of purinergic receptors in the area of the supporting cells, there may be even more layers of complexity to the activity of the supporting cells that should be further explored, as it is possible that they play an important role in modulating the chemosensory activity of the VNO.

However, the presence of purinergic receptors in the VNO suggests functional roles that may go far beyond modulation of chemosensory transduction. Purinergic receptors have been demonstrated to exert multiple functional roles in sensory systems, including controlling the development, remodeling, and regeneration of the systems (Zimmermann, 2006). In other areas of the nervous system purinergic receptors play a

role in trophic actions, regulating neuronal survival, growth, and apoptosis (Neary et al., 1996). ATP released from injured cells in the MOE leads to activation of P2 receptors on the sensory neurons, supporting cells, and basal cells, initiating protective responses and stimulating progenitor basal cell proliferation to regenerate damaged tissue (Housley et al., 2009). In other areas of the nervous system, it has been demonstrated that when nerve growth factor and P2Y receptors are co-activated, neuronal survival and growth is up-regulated (Arthur et al., 2006). In another study, activation of $P2Y_1$ and $P2Y_2$ receptors resulted in an increase of progenitor cell proliferation, leading to neurogenesis and remodeling (Zimmermann, 2006). It is possible that the P2Y receptors that have been identified in the VNO may be playing a role in the growth, development, and regeneration of the organ. Furthermore, the immunolocalization of $P2Y_1$ and $P2Y_2$ receptors in the VSNs would place them in an ideal position for this activity.

These results can serve as the basis for a wide range of future study on the topic of purinergic signaling in the VNO. To continue the line of inquiry it will be important to further identify P2 as well as P1 receptors that are present in the VNO. This study explored a few common purinergic signaling receptors, leaving many others to be investigated. The acquisition of specific antibodies to explore the immunolocalization of these receptors will be important to this process. In this study it was not possible to successfully explore the immunolocalization of the $P2X_3$ receptor due to the nonspecific nature of the antibodies available. This was unfortunate as previous studies have indicated strong immunoreactivity of $P2X_3$ in the mouse VNO (Gayle and Burnstock, 2005). Identifying and acquiring specific antibodies will be crucial to the further study of immunolocalization of purinergic receptors. With these antibodies at hand,

colocalization experiments with other cellular molecules such as $G_{a/o}$ will be possible to determine what signaling mechanisms the purinergic receptors are interacting with. In conclusion, while there is still much to be explored on this topic, this study has demonstrated that there are purinergic signaling receptors present in the mouse VNO. The immunolocalization of these receptors strongly supports the hypothesis that the receptors are playing an active role in the modulation of the chemosensory activity of the organ, and therein playing a role in the regulation of social and sexual behavior of the mammal.

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SUPPLEMENTAL DATA

Mus musculus strain mixed chromosome 3 genomic scaffold, alternate assembly Mm_Celera 232000009823021, whole genome shotgun sequence

Sequence ID: **ref|NW_001030719.1|** Length: 48261665 Number of Matches: 1
Range 1: 44826733 to 44826863

Score	Expect	Identities	Gaps	Strand	Frame
243 bits(131)	4e-62()	131/131(100%)	0/131(0%)	Plus/Plus	

Features:
P2Y purinoceptor 1

```
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Query  121       CCTATTCTCTT  131
                 ||||||||||||
Sbjct  44826853  CCTATTCTCTT  44826863
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Supplemental Figure 1. BLAST alignment results for the $P2Y_1$ receptor.

Mus musculus strain C57BL/6J chromosome 7 genomic contig, GRCm38 C57BL/6J MMCHR7_CTG11

Sequence ID: **ref|NT_039433.8|** Length: 56781936 Number of Matches: 2
Range 1: 18747664 to 18747780

Score	Expect	Identities	Gaps	Strand	Frame
206 bits(111)	8e-51()	115/117(98%)	0/117(0%)	Plus/Plus	

Features:
P2Y purinoceptor 2

```
Query  2         GGCAACAGCACGTACATGAAGTCCTCGTTGAAGCGACACTTGTATCCCAGTTCGTCCCCC  61
                 ||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||
Sbjct  18747664  GGCAACAGCACGTACTTGAAGTCCTCGTTGAAACGACACTTGTATCCCAGTTCGTCCCCC  18747723

Query  62        TCCCAGGTGCCATTGATGGTGCTATTCCAGGGTTCCAGGTCTGCTGCCATTGCCCTG  118
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18747724  TCCCAGGTGCCATTGATGGTGCTATTCCAGGGTTCCAGGTCTGCTGCCATTGCCCTG  18747780
```

Supplemental Figure 2. BLAST alignment results for the $P2Y_2$ receptor.

Mus musculus strain C57BL/6J chromosome 7 genomic contig, GRCm38 C57BL/6J MMCHR7_CTG11

Sequence ID: **ref|NT_039433.8|** Length: 56781936 Number of Matches: 2
Range 1: 18687740 to 18687864

Score	Expect	Identities	Gaps	Strand	Frame
231 bits(125)	1e-58()	125/125(100%)	0/125(0%)	Plus/Minus	

Features:
P2Y purinoceptor 6

```
Query  74        CAGACTCTCCGAGCATAGGAAAGGCTGACAGGCAGTTATGGAGCAGGACAATGGCACCAT  133
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18687864  CAGACTCTCCGAGCATAGGAAAGGCTGACAGGCAGTTATGGAGCAGGACAATGGCACCAT  18687805

Query  134       CCAGGCTCCAGGCTTGCCGCCCCACCACCTGCGTCTACCGTGAGGATTTCAAGCGACTGCT  193
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18687804  CCAGGCTCCAGGCTTGCCGCCCCACCACCTGCGTCTACCGTGAGGATTTCAAGCGACTGCT  18687745

Query  194       GCTAA  198
                 |||||
Sbjct  18687744  GCTAA  18687740
```

Supplemental Figure 3. BLAST alignment results for the $P2Y_6$ receptor.

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